REMARKS

The Office Action dated April 22, 2003 presents the examination of claims 2, 4, 6, 9, 16-19, and 21-24. Claims 14-15 and 25-26 are withdrawn from consideration. Claims 14-15 and 25-26 are canceled. No new matter is inserted into the application.

Interview

A telephonic interview was held with the Examiner on July 10, 2003. The Examiner's assistance in expediting prosecution of the present invention is appreciated.

Drawings

The Examiner notes that color photographs and color drawings are acceptable only for examination purposes. Applicants submit herewith under separate cover a petition under 37 C.F.R. § 1.84(a)(2) or (b)(2) in order to have the color photographs accepted as formal drawings. The petition is accompanied by the appropriate fee as set forth in 37 C.F.R. § 1.17(i) and three sets of photographs. An amendment to the specification referring to the color photographs is made herein. Since all of requirements for accepting color photographs are satisfied, Applicants respectfully

request that the Examiner accept the color photographs as formal drawings.

Claim Rejections under 35 U.S.C. § 103(a)

I. Claims 2, 4, 6, 16, 17, 18, 22, and 23 are rejected under 35 U.S.C. § 103(a) for allegedly being obvious over Gorski et al. (Clinical Chemistry, 43(1):193-195, 1997) in view of Maatman et al. (*Biochem. J.* 288:285-290, 1992). Applicants respectfully traverse the rejection applied to the pending claims. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

Specifically, the Examiner asserts that it would have been obvious to use the liver-type fatty acid binding protein as taught by Maatman et al. to detect specific kidney diseases relating to FABP in the method of Gorski et al. Applicants respectfully disagree. The present invention is well distinguished from these references as explained below.

(1) CHARACTERISTICS OF THE PRESENT INVENTION

The present invention is directed to a method for diagnosis or prognosis of a kidney disease in humans by detecting liver-type fatty acid binding protein (L-FABP) contained in a specimen

collected from a human. The present invention is also directed to a reagent or kit for the diagnosis or prognosis for use in the method. The cited references do not teach or even suggest such a method for diagnosis or prognosis of kidney disease, or a reagent or kit.

(2) GORSKI ET AL. AND MAATMAN ET AL. REFERENCES

Gorski et al.

Gorski et al. discloses that plasma FABP was measured in patients suffering from renal failure, and as a result, it was found that the plasma FABP concentration was significantly high. The study by Gorski et al. focused specifically on heart-type FABP as a marker for myocardial infarction. Gorski et al. fails to teach or suggest that liver-type FABP can be correlated with the diagnosis of kidney disease in humans.

Maatman et al.

Maatman et al. is concerned with the identification of FABP in human kidneys. Maatman et al. fails to teach or suggest the correlation between liver-type FABP and the diagnosis of kidney disease in humans.

It is disclosed in Maatman et al. that FABPs in human kidneys are liver-type FABP (L-FABP) and heart-type FABP (H-FABP) (cf.

Maatman et al., page 285, Abstract, lines 3-4). Maatman et al. further mentions that L-FABP may be involved in renal excretion of metabolites by binding with various ligands and may prevent nephrotoxicity of drugs by binding them, as follows:

> We can only speculate on the physiological relevance of the two FABP types in kidneys. The liver-type FABP binds various ligands and may be involved in the renal excretion of exogenous and endogenous metabolites. The liver-type FABP also binds some drugs [2,3], and may in this way prevent nephrotoxicity." (cf. page 289, right column, lines 4-9).

However, these comments by Maatman et al. are mere speculation as to the physiological relevance of L-FABP. Even though Maatman et al. speculates that L-FAPB may prevent nephrotoxicity by binding to drugs and causing their excretion from the liver, this speculation does not in any way suggest that there is a correlation between the levels of L-FABP and the diagnosis of kidney diseases. As such, it cannot be predicted from Maatman et al. that kidney disease can be diagnosed by detecting L-FABP in a human specimen.

(3) THE PRIMARY REFERENCE TEACHES AWAY FROM THE PRESENT INVENTION

(A) FABP in Gorski et al. is heart-type FABP

On page 193, Gorski et al. points out that of the so-called fatty acid-binding proteins (FABP), nine different types have been identified. Gorski et al. also points out that the FABP contained

in the heart and skeletal muscles are of the same type, i.e. "heart-type FABP." See, page 193, right column, lines 23-15 from the bottom. Gorski et al. further mentions that it is known in the art that FABP is released from the heart early after the onset of myocardial infarction. Due to its release from the heart, the concentration of plasma FABP increases. Gorski et al. finds that the concentration of FABP in the plasma is useful as a marker for myocardial infarction diagnosis. See, page 193, right column, lines 15-2 from the bottom. Thus, the skilled artisan can readily understand that Gorski et al. is directed to the study of "heart-type FABP" as a marker for myocardial infarction.

This fact is well-supported by the experiments performed by Gorski et al. For example, on page 194, middle column, second paragraph, Gorski et al. describes the experimental method as "Plasma FABP concentration was measured by a sensitive non-competitive sandwich ELISA [4]." Citation [4] refers to Wodzig et al., *Ann. Clin. Biochem.*, 34:263-268, 1997. Wodzig et al. provides the details of the ELISA method utilized by Gorski et al. This citation [4] is attached hereto as Exhibit 1. The Examiner will note that this reference has already been filed in an Information Disclosure Statement on October 3, 2002.

In Exhibit 1, Wodzig et al. mentions that a direct non-competitive (sandwich-type) ELISA was developed for the purpose of determining the heart-type fatty acid-binding protein (FABP) concentration in plasma. See, page 263, in the section labeled "Summary," lines 1-3. Further, Wodzig et al. discloses that the monoclonal antibodies (mAbs) directed against human heart-type FABP showed no cross-reactivity with human intestinal-type FABP or with human liver-type FABP. See, page 264, left column, third paragraph, subtitled "Monoclonal-antibody based assay of FABP," lines 4-7 and 13-15.

In view of this citation, the "FABP" measured by Gorski et al. was only "heart-type FABP", even though it was simply referred to as "FABP." In addition, in the passages labeled "Results and Discussion," Gorski et al. is merely referring to ("heart-type FABP.")

(B) The "heart-type FABP" of Gorski et al. is clearly different from the "liver type FABP" of the present invention

The "heart-type FABP" measured in Gorski et al. is clearly different from the "liver type FABP" measured in the present invention.

The liver-type FABP, heart-type FABP, and other types of FABPs are common in their fatty acid-binding properties and some other properties, but they are still very different proteins from each other and the homology between their amino acid sequences is only about 38 to 70%. It is also well known that they have different nucleotide sequences and exhibit differing expression distribution in tissues. For example, in humans, the liver-type FABP is expressed in liver, kidney, etc., tissues. On the other hand, the heart-type FABP is expressed in heart and skeletal muscles, and in kidney tissue. Although both FABPs are expressed in the kidney, the heart-type FABP exists mainly at the distal tubule of human kidney tissues, whereas the liver-type FAPB exists at the proximal tubule. This differential expression is shown in Example 3 and Figure 1 of the instant specification.

The following two journal articles are cited to support the fact that the heart-type FABP measured in Gorski et al. and the liver-type FABP to be measured in the present invention are clearly different: (1) Van Nieuwenhovern et al., *Lipids*, Vol. 31 Suppl: S223-S227, 1996 (attached hereto as Exhibit 2) and (2) Veerkamp et al., *Prog. Lipid Res.*, 34(1):17-52, 1995 (pages 17, 21 and 23 attached hereto as Exhibit 3). Both of these journal articles have already been made of record in the present application in an

Information Disclosure Statement (IDS). Van Nieuwenhovern et al. lists the various FABPs on page s225, Table 2. Veerkamp et al. lists the various FABPs on page 21, Table 3. In both of these tables, heart FAPB (i.e., heart-type FABP) and liver FABP (i.e., liver-type FABP) are classified as different proteins. Furthermore, Veerkamp et al. discloses the amino acid sequence alignment between human heart-type FABP and human liver-type FABP on page 23, Figure 2. It is clear from the alignment that the two FABP proteins do not share a high homology.

Accordingly, based upon the above it is clear that the heart-type FABP focused on by Gorski et al. is clearly different from the liver-type FABP to be measured by the present invention, and that the cited Gorski et al. reference does not teach or suggest that liver-type FABP may be used for diagnosis of kidney disease.

(C) Gorski et al. teaches away from using a FABP as a marker for diseases other than myocardial infarction

As noted above, Gorski et al. discloses heart-type FABP merely as a marker for diagnosis of myocardial infarction, but fails to teach or suggest the use of FABP for diagnosis of kidney diseases. In fact, Gorski et al. teaches away from using a FABP as a marker for diseases other than myocardial infarction.

In this regard, Gorski et al. mentions that low molecular-mass proteins such as heart-type FABP and myoglobin[1] are cleared mostly by the kidney, and that any change in the clearance rate of FABP would affect its concentration in plasma and thusly may lead to "erroneous interpretation." See, page 194, left column, lines 4-8 and 20-23. The skilled artisan would understand that the phrase "erroneous interpretation" means an erroneous interpretation in the diagnosis of myocardial infarction. In view of the possibility of such erroneous interpretation, Gorski et al. further states:

> As it remains an open question whether, and, if so, to what extent an insufficiency of the kidneys affects the plasma FABP concentration in patients…, we (Gorski et al.) studied plasma FABP and myoglobin in patients with chronic renal failure.

See, page 194, left column, lines 23-30. Furthermore, in the experimental results, Gorski et al. mentions that the concentration of heart-type FABP as a marker (for myocardial infarction) and myoglobin in plasma were increased in patients with chronic kidney failure. See, page 194, Table 1, and page 195, left column, lines 25-30. From this data, Gorski et al. concludes, "Our data indicate that in patients with chronic renal failure, the plasma concentrations of the biochemical markers FABP and myoglobin each are markedly increased. Thus, **caution must be taken when using**

[1] Myoglobin is hemoglobin existing in muscle fiber.

these marker proteins for early diagnosis of myocardial infarction, in case of renal insufficiency, as the preinfarct plasma concentration is very likely to be already high." See, page 195, left column, lines 25-36 [emphasis added].

As is clear from the above explanation, Gorski et al. studied the plasma concentration of heart-type FABP in patients with renal failure for the purpose of clarifying the difference in plasma FABP concentration from that of healthy persons, so that the skilled artisan could make a correct analysis **in the diagnosis of myocardial infarction**. Gorski et al. only shows this conclusion with respect to diagnosis of myocardial infarction.

Thus, Gorski et al. is merely concerned with a diagnosis of myocardial infarction using heart-type FABP as a marker, but never teaches or suggests using either FABP in the diagnosis of kidney disease. As noted above, Gorski et al. teaches away from using FABP as a marker in patients with renal insufficiency.

(4) THE CITED REFERENCES NEVER DISCLOSE NOR SUGGEST TO COMBINE L-FABP AND DIAGNOSIS OF KIDNEY DISEASE IN HUMANS

On page 6 of the Office Action, the Examiner asserts that the findings of Gorski et al. (on page 194, right column, lines 35-38) suggest that the kidney plays a dominant role in the clearance of

plasma FABP than of myoglobin. However, as noted above, these results speak to "heart-type FABP," rather than "liver-type FABP." Furthermore, although the skilled artisan would understand from this disclosure that the function of the kidney is associated with heart-type FABP plasma concentration, any person skilled in the art would also well understand that even though the molecular weights of proteins like in myoglobin and heart-type FABP are similar, the clearance of them from plasma is not necessarily similar to each other.

Accordingly, Gorski et al., even in combination with other references cited in the outstanding Office Action, does not give any motivation for substituting the heart-type FABP by another FABP, namely liver-type FABP. Further, it would be difficult, if not impossible, to predict the use of the liver-type FABP in the diagnosis of kidney disease in humans. In this regard, Applicants respectfully point out that it has been well known in the art for long time that the kidney participates in the clearance of not only heart-type FABP but also other low molecular weight proteins. This fact is also mentioned in Gorski et al. Nevertheless, a satisfactory test method suitable for the diagnosis of kidney disease has never been found.

Under these circumstances, the present inventors have

intensively studied and have newly found that the occurrence of the liver-type FABP derived from the proximal tubule of the kidney is closely correlated with kidney disease. By focusing on the liver-type FABP, a new method for the diagnosis of kidney diseases was established. The method for the diagnosis or prognosis of kidney disease according to the present invention is very important and very valuable in the determination of the most suitable therapeutic method for the treatment of kidney disease, such as steroid therapy, which is sometimes associated with undesirable side effects. Furthermore, the present invention can effectively be applied to the prognosis of kidney disease, which has hitherto been difficult by known methods. Such excellent effects of the present invention have never been taught or even suggested by the cited references.

As is clear from the above explanation, Gorski et al. merely speaks to "heart-type FABP" and not "liver-type FABP." Further, the "heart-type FABP" in Gorski et al. is treated merely as a marker for the diagnosis of myocardial infarction, rather than for the diagnosis of kidney disease in the present invention. Accordingly, Gorski et al. does not provide any motivation or suggestion to use "liver-type FABP" instead of "heart-type FABP," and then to apply "liver-type FABP" to the diagnosis of kidney disease. Since the

secondary Maatman et al. reference does not teach or suggest the correlation of L-FABP with the diagnosis of kidney disease in humans, the present invention would not be accomplished even by combining Gorski et al. and Maatman et al.

As is clear from the above, neither Gorski et al. nor Maatman et al. teach or suggest the diagnosis or prognosis of kidney disease with liver-type FABP. Hence, the present invention is not obvious over the cited references. Withdrawal of the instant rejection is therefore respectfully requested.

II-IV. Claim 9 is rejected under 35 U.S.C. § 103(a) for allegedly being obvious over Gorski et al. in view of Maatman et al., and further in view of Kimura et al. Claims 19 and 21 are rejected under 35 U.S.C. § 103(a) for allegedly being obvious over Gorski et al. in view of Maatman et al., and further in view of Galaske et al. Claims 14 and 15 are rejected under 35 U.S.C. § 103(a) for allegedly being obvious over Gorski et al. in view of Maatman et al., and further in view of Zuk et al.

Claims 14 and 15 are canceled, thus rendering rejection thereof moot. Applicants respectfully traverse the rejection applied to the pending claims. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

The remaining secondary references have no disclosure relative to the present invention. Kimura et al. discloses that male rat kidney contains heart-type FABP and a protein obtained by modifying α2μ-globulin. Galaske et al. is concerned with a model for anti-GBM nephritis and discloses a method for preparing the model for nephritis. Thus, those remaining references do not disclose or suggest the correlation of L-FABP with human kidney disease. Hence, even if those references are taken into consideration together with the disclosure of Gorski et al. and Maatman et al., a person skilled in the art would never predict the present invention.

Accordingly, the present invention is well distinguished from the cited references and is well patentable over them. Withdrawal of the instant rejection is therefore respectfully requested.

Summary

Applicants respectfully submit that the above amendments and/or remarks fully address and overcome the rejections and objections of record. The instant claims are now in condition for allowance. Early and favorable action by the Examiner is respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully

requested to contact Kristi L. Rupert, Ph.D. (Reg. 45,702) at the telephone number of the undersigned below.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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0020-4710P

Attachments: Marked-up version showing changes made
Exhibit 1: Wodzig et al., *Ann. Clin. Biochem.*, 34:263-268, 1997;
Exhibit 2: Van Nieuwenhovern et al., *Lipids*, Vol. 31 Suppl: S223-S227, 1996;
Exhibit 3: Veerkamp et al., *Prog. Lipid Res.*, 34(1):17-52, 1995

MARKED-UP VERSION SHOWING CHANGES MADE

IN THE SPECIFICATION

The following paragraph is inserted into the specification on page 8, between lines 15 and 16:

--The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.--

IN THE CLAIMS

Claims 14-15 and 25-26 are canceled.

Original Article *Ann Clin Biochem* 1997; 34: 263–268

One-step enzyme-linked immunosorbent assay (ELISA) for plasma fatty acid-binding protein

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SUMMARY. To allow a more rapid determination of heart-type fatty acid-binding protein (FABP) concentration in plasma a direct non-competitive (sandwich-type) ELISA was developed which uses high-affinity monoclonal antibodies to FABP. Total performance time of the one-step immunoassay is 45 min. The standard curve was linear between 0·2–6 μg/L, and the within-run and between-run coefficients of variations were below 6 and 11%, respectively. The serum FABP concentration measured in 79 healthy individuals was 1·6 (0·8) [mean (SD), range 0·3–5·0] μg/L. The assay can be used for rapid plasma or serum FABP measurement in the early diagnosis of acute myocardial infarction.

Additional key phrases: heart-type fatty acid-binding protein; monoclonal antibodies

Fatty acid-binding protein (FABP) is a recently introduced plasma marker of acute myocardial infarction (AMI) in man.[1-4] The plasma kinetics of FABP (15 kDa) closely resemble those of myoglobin (18 kDa) in that significantly elevated plasma concentrations are found within 3 h after AMI which generally return to normal values within 12 to 24 h.[5-7] These features make FABP a useful biochemical marker especially for the early assessment or exclusion of AMI,[5,6] and for the monitoring of a recurrent infarction.[7] Since FABP released from the heart after AMI is quantitatively recovered in plasma, FABP can also be used to estimate infarct size.[8]

As with myoglobin, small quantities of (heart-type) FABP are also found in skeletal muscle, which are released into the circulation following injury.[7] However, as the ratio of the tissue concentrations of myoglobin over FABP is different in heart (4–5) and skeletal muscles (20–70), the assessment of this ratio in plasma allows the discrimination between myocardial and skeletal muscle injury.[7]

The clinical application of FABP to confirm or exclude a diagnosis of AMI soon after admission requires a rapid test system, which is not yet available. Because FABP does not exhibit enzymatic activity, its plasma concentration has to be measured immunochemically. However, reported immunochemical assays for FABP take about 2 to 5 h to complete.[2,3,5,9] Here we describe a one-step sandwich-ELISA for FABP in plasma which uses high-affinity monoclonal antibodies, and shows a high sensitivity with a total performance time of only 45 min.

Correspondence: Jan F C Glatz PhD.

MATERIALS AND METHODS

Collection of blood samples

For determination of the reference range of FABP in serum and plasma, blood samples were withdrawn from 79 healthy subjects (61 men, median age 35, range 20–51 years; and 18 women, median age 38, range 23–51 years). Subjects were taking no medication. Blood samples were obtained between 9 and 12 am and collected in glass tubes (preparation of serum). For 10 subjects, a second blood sample was collected in glass tubes containing dry heparin (preparation of plasma). After centrifugation at 1500*g* for 10 min, serum or plasma was collected and stored at −70°C until analysis.

Comparison of the present assay with a previously described immunochemical assay for FABP was carried out on serial plasma samples from patients with confirmed AMI. We studied six randomly selected patients (five men, one woman, median age 58, range 52–61 years) from a population of 22 patients enrolled in a recent study.[10] Patients were admitted to the coronary care unit with chest pain and ST segment elevation (> 1 mm) typical of AMI within 6 h after the onset of symptoms. They received thrombolytic therapy consisting of 1·5 million units of streptokinase given by infusion in 40 min. Blood samples, obtained at admission and subsequently at nine time points (final sample was taken 24 h after admission), were collected in dry heparin-containing tubes and processed exactly as described above.

Isolation and purification of FABP

Human heart-type FABP, used as standard in the calibration curves, was purified from human heart autopsies by gel permeation and anion-exchange chromatography as described previously.[5] In addition, we used recombinant human FABP, kindly donated by Dr T Börchers (University of Münster, Germany).

Monoclonal-antibody based assay of FABP

For measurement of FABP in serum or plasma, a direct non-competitive ELISA of the antigen capture type (sandwich ELISA) was developed, based on the use of monoclonal antibodies (mAb). Thirteen mAbs, all of subtype IgG1 and directed against purified human heart-type FABP, were raised by the classical hybridoma technology and characterized by surface plasmon resonance analysis using a Pharmacia BIAcore biosensor, as described in detail elsewhere.[11] The mAbs recognized five distinct (three independent and two overlapping) epitopes on human FABP, and showed no cross-reactivity with human intestinal-type and human liver-type FABP. Seven of the mAbs were selected on the basis of stability of the hybridoma clone and affinity for FABP. Affinity constants for FABP interactions with these selected mAbs ranged from 5×10^7 to $3 \times 10^8\ M^{-1}$.[11] Antibodies were stored in buffer solution at −20°C, and conjugated antibodies at 4°C, and were found to be stable for more than 12 months.

Assays were carried out on polyvinylchloride microtitre plates (Falcon type 3912; Becton Dickinson, Oxnard, CA, USA). Plates were coated overnight at 4°C with 200 ng per well of a specific mAb in 100 μL 0·1 M sodium bicarbonate buffer (pH 9·4). The wells then were washed five times with phosphate-buffered saline containing 0·1% (w/v) bovine serum albumin (A4503 from Sigma, St Louis, MO, USA) and 0·05% (v/v) Tween-20 (PBT). Thereafter, 50 μL of a solution containing 180 ng/mL of mAb conjugated with horseradish peroxidase (HRP; P8375 from Sigma, St Louis, MO, USA) and recognizing a different epitope group on FABP was added to each well. This was immediately followed by 50 μL of the standard containing 0–12 μg/L FABP (0–600 pg purified human FABP per well), or 50 μL of 25-fold prediluted human serum or plasma samples (fivefold predilution in case of healthy subjects). After incubation for 30 min at 37°C the microtitre plates were washed five times with PBT and developed with 100 μL per well of a substrate solution consisting of 20 mmol/L tetramethyl benzidine (TMB) and 6 mmol/L H_2O_2 dissolved in 0·1 M sodium citrate buffer (pH 5·0). After 5 min, the reaction was stopped with 50 μL of 2 M H_2SO_4 per well, and the absorbance was read at 450 nm using a Titertek Multiscan MKII microplate reader. The detection limit of the assay was 0·2 μg/L (20 pg/well).

Polyclonal-antibody based assay of FABP

The newly developed mAb based sandwich ELISA was compared with the polyclonal-antibody (pAb) based ELISA described previously.[5] The latter assay was carried out in a similar manner as outlined above, but used monospecific polyclonal IgG antibodies isolated by affinity chromatography from rabbit serum. After coating the wells with capture antibody, blocking with 5% (w/v) bovine serum albumin, and addition of standards or diluted plasma samples, incubation took place for 1·5 h at room temperature. The plates then were washed five times with PBT and incubated for another 1·5 h with 60 ng/well biotinylated monospecific polyclonal rabbit IgG (detector antibody) in 100 μL of PBT. Thereafter, the plates were washed again and detection of the biotinylated antibody bound to FABP was achieved by incubation with 100 μL of streptavidine-HRP (Pierce, Rockford, IL, USA) for 1 h, followed by washing and development with 100 μL per well of a substrate solution consisting of 20 mmol/L O-phenylene diamine (OPD) and 6 mmol/L H_2O_2 in 0·1 M sodium citrate buffer (pH 5·0). After 5 min, the reaction was stopped with 50 μL

of 2 M H_2SO_4 and the absorbance was read at 492 nm. Detection limit of the assay was 1 μg/L (50 pg/well).[5]

Statistical analysis

Data are presented as mean (SD). Statistical analysis of differences between groups was performed with Student's *t*-test. The level of significance was set at $P<0{\cdot}05$.

RESULTS AND DISCUSSION

Performance of monoclonal antibody-based assay

The seven different mAbs selected on the basis of stability of the hybridoma clone and affinity for FABP were tested both as capture and as detector antibodies (the latter conjugated with HRP) in different combinations by a checkerboard approach. For combinations of mAbs directed against distinct epitope groups a similar linear FABP standard curve was found, with the absorbance at a specific FABP concentration differing maximally twofold from that observed with the eventually selected mAbs. Optimal results were obtained with the combination of mAb 67D3 immobilized on the polyvinylchloride microtitre wells (capture antibody) and mAb 66E2 conjugated with HRP as detector antibody. The standard curve was found to be linear up to 6 μg/L (600 pg/well) FABP, and no differences were found between a standard curve recorded with FABP isolated from human heart and that recorded with recombinant human FABP (the absorbance with the latter was 97±5% ($n=3$) (Fig. 1). The linearity of the standard curve would allow reducing the number of standards to two or even one, thus facilitating the use of this assay for patient samples. Reduction of the incubation time from 30 to 15 min did not affect the linearity of the standard curve, but increased the intra-assay variability to above 10% so that the routine incubation time was set at 30 min (data not shown).

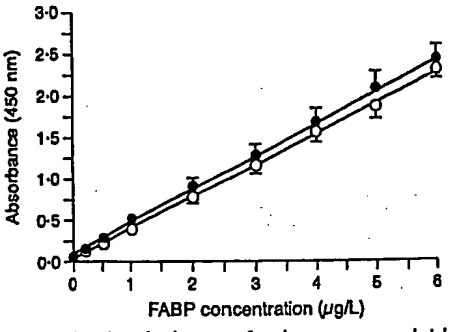

FIGURE 1. *Standard curves for the one-step sandwich enzyme-linked immunoasborbent assay (ELISA) for heart-type fatty acid-binding protein (FABP), using monoclonal antibodies and tissue-derived (●) or recombinant (○) human heart FABP. Data refer to mean (SD) for 10 curves. For the lower values the SD is not larger than the size of the symbols used.*

Assay variation and linearity of dilution

To estimate the imprecision of the assay two different human plasma pools with elevated FABP concentrations were analysed three times each on 10 consecutive days. For the pooled plasma sample with a relatively low FABP concentration (26 μg/L) the calculated intra-assay coefficient of variation (CV) was 6%, and the inter-assay CV 11%; for the pooled plasma sample with a high FABP concentration (280 μg/L) the intra-assay CV was 5% and the inter-assay CV 9%.

The linearity of dilution was investigated using the plasma samples with a high FABP concentration. Serial dilutions (up to 10-fold) were made in saline. After correction of the measured value for the dilution factor, recovery was calculated to be between 97 and 113%. Recovery of pure human FABP added in various quantities to plasma of healthy individuals amounted to 97 (5)% [mean (SD) for 12 determinations].

Method comparison

Fifty-four plasma samples with FABP concentrations between 2 and 310 μg/L, as assessed with the existing immunochemical assay based on polyclonal antibodies, were re-evaluated using the newly developed mAb ELISA. The correlation found was $y=1{\cdot}04x+0{\cdot}23$ ($r=0{\cdot}99$). Agreement between the two methods was analysed in a difference plot according to Bland and Altman (Fig. 2).[12] The accuracy was assessed by calculating the 95% confidence interval [mean (2 SEM)] for the mean of the differences (4·3 μg/L). As this interval (−0·8–7·8 μg/L) includes zero, there is no evidence of systematic bias. With respect to the precision, the scatter of the differences was found to increase as the average increased (Fig. 2), but the limits of agreement [mean (2 SD) i.e. −21·5 and +30·2 μg/L] are acceptably small. We conclude that there is agreement between the methods.

To determine the stability of FABP in plasma samples on storage and following repeated cycles of freezing and thawing, six plasma

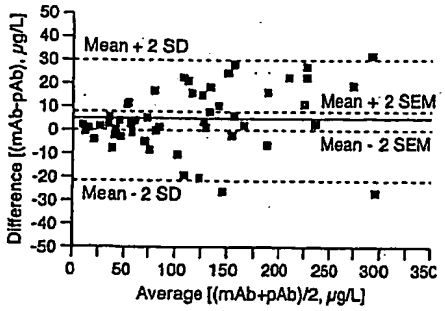

FIGURE 2. *Difference plot of 54 plasma samples in which FABP concentration was measured by monoclonal antibody-based (mAb) and polyclonal antibody-based (pAb) immunochemical assay. Mean difference (solid line), 95% confidence interval for the mean difference (mean (2 SEM) dashed line), and limits of agreement (mean (2 SD) dashed line) are shown.*

samples with FABP concentrations ranging from 25 to 250 µg/L were assayed freshly, then divided into aliquots and stored at 4°C for 1 week, and at −20°C and −70°C for 12 months. When compared to fresh plasma samples, no significant alterations in FABP concentration were found in these samples as analysed in the given time periods. Repeated freezing and thawing (tested up to eight times) also had no influence on measured FABP concentrations (data not shown). The high stability of FABP in plasma agrees with observations made by Ohkaru *et al.*[9]

Reference values

The mAb ELISA was used to measure the FABP concentration in serum and heparinized plasma samples from 79 apparently healthy individuals (Table 1). Men showed a slightly higher concentration of FABP [1·6 (0·8) µg/L, mean (SD) for $n=61$) than women [1·4 (0·5) µg/L, mean (SD) for $n=18$), but the difference was not significant. FABP concentrations measured in serum did not differ from those measured in heparinated plasma [plasma/serum ratio 1·04 (0·08) mean (SD), $n=10$].

TABLE 1. *Fatty acid-binding protein (FABP) concentration in serum samples from healthy subjects*

Sex	Age	n	FABP concentration (µg/L) Mean (SD)	Range
Women	20–30	5	1·0 (0·6)	0·3–1·7
	31–40	8	1·6* (0·4)	1·0–2·2
	41–51	5	1·6 (0·6)	0·7–2·4
	All	18	1·4 (0·5)	0·3–2·4
Men	20–30	19	1·2 (0·4)	0·5–2·0
	31–40	30	1·8* (0·9)	0·9–5·0
	41–51	12	1·8* (0·9)	0·9–3·7
	All	61	1·6 (0·8)	0·5–5·0

*Significantly different from value at age 20–30 ($P<0·05$).

Serum FABP showed a slight increase with age with, for instance, the mean values found for men and women of 41–51 years being 60% and 50% higher, respectively, than in men and women of 20–30 years (Table 1); these differences are significantly different ($P<0·05$). The increase of serum FABP with age most likely relates to the fact that FABP is eliminated from the circulation predominantly by renal clearance[3,5] and that renal function generally decreases with age.[13] In addition, release of FABP from muscle tissue may increase with age. For plasma myoglobin, which is also cleared from the circulation mostly by the kidneys,[14] a similar increase with age has been found.[15,16]

For all subjects investigated the mean (SD) value of serum FABP was 1·6 (0·8) µg/L ($n=79$). This value agrees with studies of Tanaka *et al.*[3] who reported FABP in plasma from healthy subjects to vary between 0 and 2·8 µg/L ($n=86$), although recently the same investigators reported a higher reference concentration [3·6 (1·8) µg/L, mean (SD), $n=100$].[9] Our present value, however, is lower than that determined previously in our laboratory [9 (5 µg/L), $n=72$].[5] The latter difference may relate to the use in the ELISA at that time of less specific polyclonal antibodies when compared to the present monoclonal antibodies, which would lead to overestimated values in case of low predilution of samples (fivefold predilution was used). The lower sensitivity of the previous assay may also have contributed to this difference.

Evaluation

A non-competitive sandwich ELISA for FABP was developed which is based on the use of monoclonal capture and detector antibodies recognizing different epitope groups on the protein molecule. This permits the simultaneous incubation of the FABP-containing serum or plasma sample with the (immobilized) capture antibody and the (conjugated) detector antibody, thus limiting the number of steps of the assay procedure. In addition, an incubation time of 30 min appeared sufficient, which relates to

the antibodies having been selected for a high affinity for FABP. Since detection of the HRP conjugate is very rapid (<7 min), the entire procedure, including washing steps and reading of the absorbance, can be performed routinely within 45 min, which is markedly faster than the 5 h needed to complete the previously developed polyclonal antibody-based immunochemical assay.[5]

The newly developed immunoassay showed a low intra-assay and inter-assay variation and an excellent comparability with the previously described assay for FABP.[5] The detection limit (0·2 μg/L) is markedly lower than that of reported direct[5,9,17] or competitive immunoassays,[2,3] and is sufficiently low to permit the accurate determination of FABP concentrations in plasma from healthy individuals. The measuring range allows samples from patients with AMI to be routinely diluted 10-fold (20-fold final dilution in the assay) and include >90% of reported FABP concentrations in pathological samples (4–120 μg/L).[2,3,5–8] The use of monoclonal antibodies ensures a source of antibodies of constant quality so that the assay can be easily automated. In view of the good performance of FABP as plasma marker in the early phase diagnosis of acute myocardial infarction, the immunoassay described here may well be used for determination of FABP in blood from patients entering the coronary care emergency room of the hospital, and will also be useful as reference assay for even more rapid assay systems such as an FABP immunosensor currently being developed.[18]

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Membrane-Associated and Cytoplasmic Fatty Acid-Binding Proteins

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ABSTRACT: A number of cellular fatty acid-binding proteins are being implicated in the uptake and intracellular transport of long-chain fatty acids by parenchymal cells. Having been a topic of research for more than 20 years, cytoplasmic fatty acid-binding proteins now are assigned various pivotal functions in intracellular fatty acid transport and metabolism. More recently several membrane-associated fatty acid-binding proteins have been identified and these proteins are thought to function in the transmembrane transport of fatty acids. In this review, a short summary is provided of the latest developments in this research area.

Lipids 31, S-223–S-227 (1996).

Long-chain fatty acids (FA) are important compounds serving as substrates for energy-production and formation of phospholipids (1). Moreover, they can participate in signal transduction pathways (2–4). Adipose tissue takes up large amounts of FA from blood plasma for storage as intracellular triacylglycerols. In contrast, oxidative skeletal muscles and especially the heart take up FA as fuel for contractile processes. Since FA are poorly soluble in water the rapid metabolism of these FA would probably not be possible without special binding proteins, which dramatically increase the solubility of these compounds in aqueous environments. In blood and interstitium, FA are mainly bound to albumin, keeping the unbound FA concentrations extremely low (<10 nM) (5). Intracellularly, FA are most likely bound to specific cytoplasmic fatty acid-binding proteins (FABP) (6–10). More recently, membrane-associated proteins have been hypothesized to function in the cellular uptake of FA. These membrane and cytoplasmic proteins will be discussed in more detail below.

Membrane-associated FABP. The mechanism of transmembrane transport of FA into parenchymal cells has been a matter of debate for several years. Some investigators favor a mechanism in which FA cross the membrane by simple diffusion (11,12). Others found saturable uptake of FA in several cell-types, a process that could be inhibited by FA analogues, indicating that membrane-associated proteins are involved (13). Subsequently, by using various techniques, a number of such membrane proteins have been identified (Table 1). The first membrane protein described to be involved in the uptake of FA is a 40 kDa protein present in the plasma membrane of rat liver cells (14). This protein, $FABP_{PM}$, was subsequently also found in intestine (15), and heart (16). The protein seems closely related, if not identical, to mitochondrial aspartate aminotransferase (17). $FABP_{PM}$ is not an integral membrane protein but has been shown to possess affinity for membranes (17).

In 1987, Fujii *et al.* (18,19) described another membrane protein with high affinity for FA, which was designated fatty acid receptor (FAR). This protein of about 60 kDa was found to be present in kidney and heart. The third membrane protein putatively involved in FA uptake, identified in adipocytes by Harmon and co-workers (20) and recently cloned by Abumrad *et al.* (21), is an 88 kDa protein called fatty acid translocase (FAT). FAT from rat was found to be highly homologous (85%) to the human leucocyte differentiation antigen CD36, a receptor protein present among others on monocytes and platelets, and thought to be involved in adhesion phenomena and intracellular signalling (for review on CD36 see ref. 22). Comparison of rat FAT (21) and the recently cloned mouse CD36 (23) revealed an amino acid identity of 93%, which strongly suggests that these proteins are species homologues. Recent investigations showed that CD36 is an integral membrane protein with one transmembrane region (24).

In adipocytes, Trigatti *et al.* (25) found another membrane protein implicated in the transmembrane transport of FA. This 22 kDa protein was identified by photoaffinity labelling with a FA analogue. The final membrane protein hypothesized to augment cellular FA uptake that has been found up till now, is called fatty acid transport protein (FATP) (26). A functional approach using expression cloning was followed to identify this 63 kDa protein in adipocytes. FATP was shown to be an integral membrane protein present in the plasma membrane of several tissues, and has been predicted to have several membrane spanning domains (26).

The precise functions of the FA-binding membrane-associated proteins in the transport of FA across cellular membranes are still unclear. Some of the possible mechanisms by which membrane proteins could be involved in this process

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Abbreviations: FA, long-chain fatty acids; FABP, fatty acid-binding protein; FAR, fatty acid receptor; FAT, fatty acid translocase; FATP, fatty acid transport protein.

TABLE 1
Membrane-Associated Fatty Acid-Binding Proteins (FABP)

Protein	Molecular mass (kDa)	Major occurrence	Reference
Membrane FABP	22	Adipose tissue	25
$FABP_{PM}$	40	Liver, heart, adipose tissue, intestine	15,16
FA receptor	56–60	Heart, kidney	18,19
FA transport protein	63	Adipose tissue, heart, skeletal muscle	26
FA translocase	88	Adipose tissue, heart, skeletal muscle	20,21

are shown in Figure 1. These proteins might function as a translocator, but it is also possible that they represent acceptors for FA released from albumin and that FA subsequently cross the plasma membrane by diffusion through the phospholipid bilayer. Albumin binding proteins have been described which could play a role in this mechanism (27). Furthermore, there might be a direct interaction between a membrane-associated FA-binding protein and (extracellular) albumin and/or (intracellular) FABP. Such interactions would ensure a controlled uptake mechanism in which FA remain protein-bound. Further characterization of the FA-binding membrane proteins will provide a better understanding of the complex transmembrane transport mechanisms of FA.

Intracellular FABP. Cytoplasmic FABP belong to a single gene family of intracellular lipid binding proteins of 14–15 kDa, capable of binding hydrophobic ligands with high affinity (28). The mammalian cytoplasmic lipid-binding proteins are listed in Table 2. Since the discovery of FABP in 1972 by Ockner and co-workers (29), nine different FABP, including the recently discovered testicular 15 kDa protein (T-LBP) (30,31), have been identified (Table 2). All lipid binding proteins are composed of two α-helices and 10 anti-parallel β-strands, organized in two β-sheets, thereby forming a clam shell-like structure (8). The lipid ligand is bound inside the molecule by interaction with specific amino acid residues within the binding pocket of the lipid binding protein (8). A recent investigation by Richieri *et al.* (32) suggests that the FABP have a higher affinity for the binding of FA than found in previous studies (7,9,10) with the dissociation constants (Kd) ranging from 2 to 1000 nM, depending on type of FABP and FA (32). A striking feature of the FABP is their relative abundance in tissues with active FA metabolism.

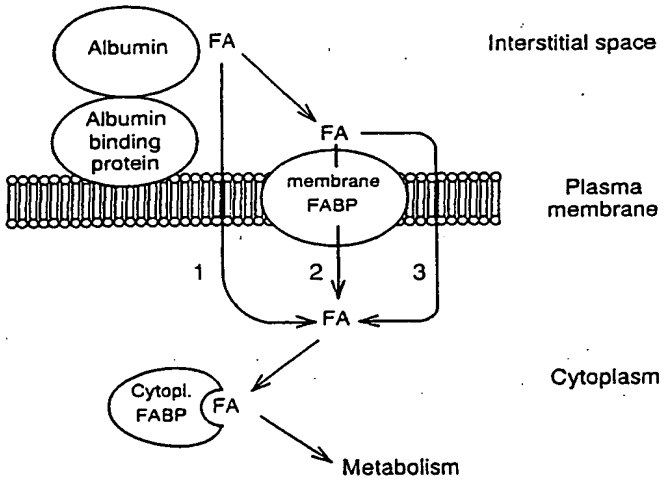

FIG. 1. Schematic presentation of the mechanism of cellular fatty acid uptake and the possible role of membrane-associated fatty acid-binding proteins in this process. Transmembrane transport could take place without the involvement of membrane proteins (1), or with a membrane protein acting as a true translocator (2), or as an acceptor for fatty acids thus, creating a steeper transmembrane gradient (3). FA, long-chain fatty acid; membrane FABP, any membrane-associated fatty acid-binding protein (see Table 1); cytopl. FABP, any cytoplasmic FABP (see Table 2).

A number of biological roles have been ascribed to the FABP: (i) Facilitation of the transport of FA to their intracellular sites of utilization. Indirect evidence was provided in studies showing a correlation between FA-utilization and FABP-content (33,34) and by showing that *in vivo* FA are bound to FABP (35,36). Furthermore, *in vitro* studies showed an increase in FA transport from isolated mitochondria to artificial phospholipid vesicles in the presence of H-, or L-FABP (37). Theoretical studies also support a role for FABP in FA-transport in cardiomyocytes (38). More direct evidence was found by transfection studies in which cells transfected with H-, L-, or A-FABP showed increased FA-uptake rates (39-41). Finally, a recent report showed that a single amino acid substitution (Ala54 -> Thr54) in human I-FABP, as occurs in some Pima Indians, causes a two-fold greater affinity for FA. This was associated with increased fat oxidation rates and with insulin resistance, also suggesting a role for I-FABP in cellular FA-transport (42). (ii) Prevention of local high FA concentrations and thereby protecting the cell against detrimental effects of FA, for instance in the ischemic heart (43). (iii) By modulating hydrophobic ligand metabolism, FABP can influence important cellular events like mitogenesis (44) and FA-mediated signal transduction pathways (3,7). Recently, a small fraction of H-FABP in rat heart and mammary gland was found to be phosphorylated upon stimulation with insulin, and it was suggested that H-FABP might play a role in signal transduction downstream from the insulin receptor (45,46). (iv) Inhibition of growth and induction of differentiation of cultured mammary epithelial cells has been reported as an extracellular function of mammary derived growth in-

TABLE 2
Mammalian Cytoplasmic Lipid-Binding Proteins[a]

Protein	Current designation	Other designations	Ligand	Major occurrence
Heart FABP	H-FABP	MDGI	FA	Heart, skeletal muscle, smooth muscle, brain, kidney, mammary gland
Liver FABP	L-FABP	Z-protein	FA, heme, bilirubin, prostaglandins	Liver, small intestine, kidney
Intestinal FABP	I-FABP		FA	Small intestine
Adipocyte lipid-binding protein	A-LBP	A-FABP, aP2	FA, retinoic acid	Adipose tissue
Epidermal FABP	E-FABP		FA	Epidermis
Brain FABP	B-FABP		FA	Nervous system
Myelin FABP	M-FABP	Myelin-P2	FA, retinoids	Nervous system
Ileal lipid-binding protein	I-LBP	I-BABP, gastrotropin	FA, bile acids	Small intestine (ileum)
Testicular lipid-binding protein	T-LBP	PERF15	ND	Testis
Cellular retinoic acid-binding protein	cRABP		Retinoic acid	Testis, nervous system, kidney, skin
Cellular retinoic acid-binding protein II	cRABPII		Retinoic acid	Skin, adrenals
Cellular retinol-binding protein	cRBP		Retinol	Widespread expression
Cellular retinol-binding protein II	cRBPII		Retinol	Intestine

[a]Data are compiled from References 3, 8, 30, 31, and 56. ND, not determined; FA, long-chain fatty acids, other abbreviation as in Table 1.

hibitor, which is identical to H-FABP (9). (v) Induction of myocyte hypertrophy through binding with a high affinity receptor is another extracellular function recently ascribed to H-FABP (47).

Regulation of expression of FABP. The expression of A-FABP and also of FAT and FATP is upregulated during differentiation from preadipocytes to adipocytes (21,26,48), and is paralleled or preceded by an increased uptake of FA (48).

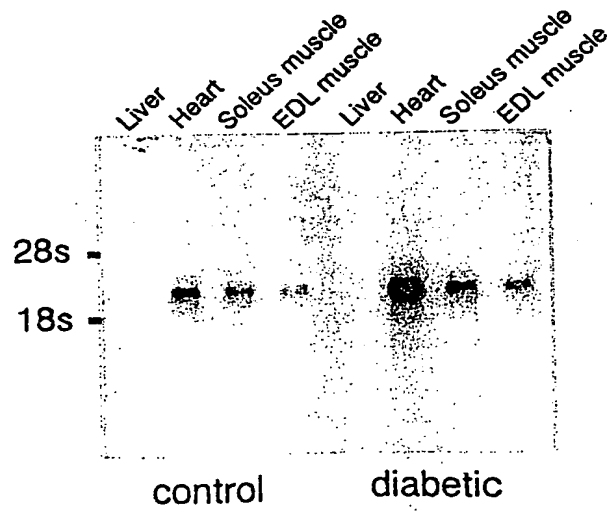

FIG. 2. Northern blot analysis of total RNA from liver, heart, soleus, and extensor digitorum longus (EDL) muscle from control and streptozotocin-induced diabetic rats using fatty acid translocase cDNA as probe.

Recently, it was shown that FA themselves can induce the expression of A-FABP in rat preadipocytes (49).

In cardiac cells, H-FABP expression increases markedly during the first few days after birth and in the weaning period (50,51). During this period, the FA oxidation capacity is also increased (52). Recently, it was shown that FAT is co-expressed with H-FABP in rat muscles and that FAT expression followed a similar upregulation in heart during development, indicating related biological functions (53).

Diabetes is a disease in which several organs, like the heart, rely more on FA oxidation as their major energy source. In streptozotocin-induced diabetic animals, a decrease of A-FABP expression (54) and an increase in H-FABP expression (55) have been shown. These results indicate that insulin deficiency can influence FABP expression levels. Preliminary results from our laboratory show that FAT is also upregulated in rat muscles in streptozotocin-induced diabetic rats (Fig. 2). These results suggest that altered FA-handling of cells can influence FABP and FAT expression.

Concluding remarks. In the last 20 years, a number of cellular proteins with affinity for FA have been identified. Among these are nine different types of intracellular 15 kDa FABP which most likely function in facilitating the *trans*-cytoplasmic transport of FA. In the last few years, more interest in the transmembrane transport of FA has resulted in the discovery of membrane proteins that might be involved in this process. Now that the genes of most of these proteins have been cloned, molecular biological techniques can be applied to manipulate the cellular content and binding activity of

these proteins. This might provide a better understanding of their role in the complex mechanisms by which FA are taken up and distributed in the cell.

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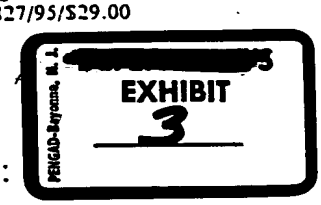

CYTOPLASMIC FATTY ACID-BINDING PROTEINS: THEIR STRUCTURE AND GENES

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CONTENTS

I. INTRODUCTION

Lipid-binding, -transfer or -exchange proteins are present in intra- and extracellular fluids of all organisms. They play a role in the transport or targeting of lipids in the cell or in the plasma, but may also interact directly or indirectly by modulation of the free ligand concentration with various cellular processes. Some lipid-binding proteins are rather specific, others bind various hydrophobic ligands, e.g. serum albumin, non-specific lipid transfer protein, liver fatty acid-binding protein (FABP). Lipid-binding molecules belong to several unrelated families of proteins and many molecules have not been characterized well. The structure of three families of lipid-binding proteins has been established (Table 1). Many of these proteins bind fatty acids as their main ligand, but also proteins with a quite different structure have affinity to fatty acids (Table 2). Before we come to the main subject of this review, the cytoplasmic fatty acid-binding proteins (FABPs) and the other members of the FABP family, we will first shortly discuss other proteins which bind fatty acids.

Albumin is the main transporter of free fatty acids in the blood,[318] but in the fetal blood α-fetoprotein and fetuin are also involved in fatty acid transport.[342] Serum vitamin D-binding protein has a low affinity for fatty acids.[53] The structure of the fatty acid-binding sites of albumin[319] and human α-fetoprotein[236] have been described. Lactoglobulin is the only member of the lipocalins (Table 1) which has a rather high affinity for fatty acids,[320,264] but also binds retinol.[264] Heat-shock protein (72 kDa) contains 2 molecules of nonesterified palmitic and oleic acid each per isolated dimer.[109] Glutathione *S*-transferases bind fatty acid in their regulatory domain II at the C-terminal side.[184,235] Other proteins which bind fatty acids are the membrane fatty acid-binding proteins found in *Escherichia coli*[37] and in the plasma membrane of various mammalian cell types as adipocytes, enterocytes, hepatocytes and myocytes.[262,315] We will discuss these proteins below.

retinol distribution between serum retinol-binding protein and CRBP is at equilibrium.[238] Evidence for the involvement of FABP in fatty acid uptake on basis of physiological data and results with inhibitors of fatty acid binding to FABP is equivocal,[374] but recently FABPs of Hep G2 cells, primary rat hepatocytes and differentiated 3T3 adipocytes were labeled in a time- and temperature-dependent fashion at the uptake of a photoactivable radioiodinated fatty acid analogue.[328,359,381,382] Transport of fatty acids from and to natural and model membranes has been observed for different types of FABPs.[63,163,164,209,253,330–332,397] The transfer function of FABP was also demonstrated in a model cytosol system.[326] The surface charge on the FABP and on the membrane of the cellular organelles may influence the transfer rate from FABP to membrane.[119,397]

With laser photobleaching the intracellular transport of a fluorescent fatty acid analogue was characterized in cultured hepatocytes.[192] The cytoplasmic diffusion rate and the fraction of cellular fluorescent fatty acid in aqueous cytoplasm was larger in female than male cells corresponding with their difference in FABP content.[23,152,240,248] The investigators suggested that FABP and other cellular binding proteins may enhance the diffusive flux of their ligand by reducing membrane binding.[192]

III. DISTRIBUTION AND STRUCTURE OF CYTOPLASMIC FATTY ACID-BINDING PROTEINS (FABPs)

A. *Distribution*

FABPs have been isolated from cytosols of tissues of vertebrates and invertebrates by various procedures, including gel filtration, ion-exchange and affinity chromatography, precipitation with salt or organic solvents, and preparative electrophoresis.[249] The purification methods had to be adapted for different tissues, not only due to different contaminating proteins. Tissue-specific FABP types appeared to exist, which were named after the first tissue of isolation. At the moment at least seven FABP types have been established on base of amino acid and/or cDNA sequence (Table 3). They show a characteristic tissue and cellular distribution. The presence of a certain FABP type is mostly established by isolation of the protein or by Western and/or Northern blots, but in some tissues the immunochemical or immunohistochemical analyses need additional evidence.

In some tissues a certain FABP type is limited to specific cell species, e.g. liver FABP to hepatocytes.[20,89,130,346,347] In other tissues more FABP types are present in different or similar cell types. Four FABP types are expressed in the stomach, depending on the cell type and the developmental stage.[4,129] The liver and heart FABP types are present in the kidney, but at different locations.[194,195] In intestine both the liver and intestinal FABP types are found in enterocytes of jejunum and ileum, but in colonocytes only the liver FABP

TABLE 3. Tissue Occurrence of FABP Types*

Liver type‡	Liver,† intestine,† kidney,† stomach
Intestinal type	Intestine,† stomach
Heart type	Heart,† kidney,† skeletal muscle,† aorta,† Adrenals,† placenta,† brain,† testes, ovary, Lung, mammary gland, stomach
Adipocyte type§	Adipose tissue†
Myelin type‖	Peripheral nervous system†
Ileal type¶	Intestine,† ovary, adrenals, stomach
Epidermal type	Skin†

*The indication of a FABP type in a tissue does not mean its presence in all cell types of that tissue; the FABP type may be limited to specific cells or may be present at certain developmental stages.

†Evidence was obtained both by protein and mRNA analysis.

‡Previously termed Z-protein or aminoazo dye-binding protein A or wrongly sterol carrier protein.

§Also named adipocyte lipid-binding protein or initially p422 or aP$_2$.

‖Originally named myelin P$_2$ protein.

¶Also named ileal lipid-binding protein and originally gastrotropin.

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                1
L-FABP  MS....FSGK YQLQSQENFE AFMKAIGLPE EL..IQKGKD IKGVSEIVQN 43
I-FABP  MA....FDST WKVDRSENYD KFMEKMGVNI VKRKLAAHDN LK..LTITQE 43
H-FABP  MVDA..FLGT WKLVDSKNFD DYMKSLGVGF ATRQVASMT. .KPTTIIEKN 45
My-FABP .SNK..FLGT WKLVSSENFD DYMKALGVGL ATRKLGNLA. .KPTVIISKK 45
A-FABP  MCDA..FVGT WKLVSSENFD DYMKEVGVGF ATRKVAGMA. .KPNMIISVN 45
E-FABP  MATVQQLEGR WRLVDSKGFD EYMKELGVGI ALRKMGAMA. .KPDCIITCD 47
Il-FABP MA....FTGK YEIESEKNYD EFMKRLALPS DA..IDKARN LKIISEVKQD 43
CRBPI   MPVD..FTGY WKMLVNENFE EYLRALDVNV ALRKIANLL. .KPDKEIVQD 45
CRBPII  MTKD..QNGT WEMESNENFE GYMKALDIDF ATRKIAVRL. .TQTKIIVQD 45
CRABPI  MPN...FAGT WKMRSSENFD ELLKALGVNA MLRKVAVAAA SKPHVEIRQD 46
CRABPII MPN...FSGN WKIIRSENFE ELLKVLGVNV MLRKIAVAAA SKPAVEIKQE 46

L-FABP  GKHFKFTITA GSKVIQNE.F TVGEECE..L ETMTGEKVKT VVQLEGDNKL 90
I-FABP  GNKFTVKESS AFRNIEVV.F ELGVTFN..Y NLADGTELRG TWSLEGNKLI 90
H-FABP  GDILTLKTHS TFKNTEIS.F KLGVEFDE.. TTADDRKVKS IVTLDGGKLV 92
My-FABP GDIITIRTES TFKNTEIS.F KLGQEFEE.. TTADNRKTKS IVTLQRGSLN 92
A-FABP  GDVITIKSES TFKNTEIS.F ILGQEFDE.. VTADDRKVKS TITLDGGVLV 92
E-FABP  GKNLTIKTES TLKTTQFS.C TLGEKFEE.. TTADGRKTQT VCNFTDGALV 94
Il-FABP GQNFTWSQQY PGGHSITNTF TIGKECD..I ETIGGKKFKA TVQMEG.GKV 90
CRBPI   GDHMIIRTLS TFRNYIMD.F QVGKEFEEDL TGIDDRKCMT TVSWDGDKL. 93
CRBPII  GDNFKTKTNS TFRNYDLD.F TVGVEFDEHT KGLDGRNVKT LVTWEGNTLV 94
CRABPI  GDQFYIKTST TVRTTEIN.F KVGEGFEE.. ETVDGRKCRS LATWENENKI 93
CRABPII GDTFYIKTST TVRTTEIN.F KVGEEFEE.. QTVDGRPCKS LVKWESENKM 93

L-FABP  VTTFKNIKSV ......T.EL .NGDIITNTM TLGDIVFKRI SKRI. 126
I-FABP  ...GKFKRTD NGNELNTVRE IIGDELVQTY VYEGVEAKRI FKKD. 131
H-FABP  H....LQKWD GQETTLVREL I.DGKLILTL THGTAVCTRT YEKEA 132
My-FABP Q....VQRWN GKETTIKRKL V.DGKMVAEC KMKGVVCTRI YEKV. 131
A-FABP  H....VQKWD GKSTTIKRKR E.DDKLVVEC VMKGVTSTRV YERA. 131
E-FABP  Q....HQEWD GKESTITRKL K.DGKLVVEC VMNNVTCTRI YEKVE 134
Il-FABP VVNSPNYHH. ......TAEI V.DGKLVEVS TVGGVSYERV SKKLA 127
CRBPI   ...QCVQKGE KEGRGWT.QW IEGDELHLEM RVEGVVCKQV FKKVQ 134
CRBPII  C....VQKGE KENRGWK.QW VEGDKLYLEL TCGDQVCRQV FKKK  133
CRABPI  HCTQTLLEGD GPKTYWTREL AND.ELILTF GADDVVCTRI YVRE. 136
CRABPII VCEQKLLKGE GPKTSWTREL TNDGELILTM TADDVVCTRV YVRE. 137
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Fig. 2. Alignment of the amino acid sequences of the members of the FABP family. All sequences are for human proteins, except ileal FABP and CRBP II, respectively for pig and rat. Identical residues present in at least 5 molecules are shaded.

to impede fatty acid binding to adipocyte FABP.[52] On the other hand, fatty acid binding activates phosphorylation of this protein.[124] Insulin receptor tyrosine kinase phosphorylated only less than 0.1% of human muscle FABP in *in vitro* experiments. (Prinsen and Veerkamp, unpublished data). Delipidation or fatty acid loading had no effect. No phosphorylation of FABP was observed with or without insulin stimulation in incubated rat soleus muscle or cultured rat muscle cells. The physiological relevance of tyrosine phosphorylation of FABP is therefore doubtful.

Heterogeneity of protein preparations (immunologically pure) has been found at chromatography and isoelectric focussing for various FABP types, especially for liver FABP.[208,352,374] Many possible causes have been suggested, as fatty acid loading, glutathione-FABP mixed disulfide formation, partial unfolding, N-acetylation, amidation of acidic residues. Recently it was established that amino acid exchange (Asp^{105}–Asn^{105}) and covalent modification by cysteine and glutathione could explain the heterogeneity of FABP from bovine liver.[81] The modifications did not affect fatty acid binding. Only one of two cytoplasmic isoforms of heart FABP was found in mitochondria of bovine heart.[306]

A high degree of similarity exists between the same FABP, CRBP or CRABP type from different mammalian species.[9,374] About the primary structure of members of FABP family in other vertebrates no data are available, although they were also isolated from various tissues of chicken and fish.[374] Muscle FABPs from two locusts, *Schistocerca gregaria*[116,263] and *Locusta migratoria*[193] show 41 and 42% identity of amino acid sequence with human muscle FABP[254] and a high percentage of conservative substitution, especially in residues involved in fatty acid binding.[193] The flatworm, *Schistosoma mansoni* contains a 15 kDa